

# Dietary soya saponin improves the lipid metabolism and intestinal health of laying hens

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**ABSTRACT** Soya saponin (SS) is a natural active substance of leguminous plant, which could improve lipid metabolism and regulate immune function. Intestinal flora might play a key role in the biological functions of SS. The objective of this study was to measure the effects of dietary SS on immune function, lipid metabolism, and intestinal flora of laying hens with or without antibiotic treated. The experiment was designed as a factorial arrangement of 3 dietary SS treatments × 2 antibiotic treatments. Birds were fed a basal diet (CON) or a low-SS diet (50 SS) containing 50 mg/kg SS, or a high-SS diet (500 SS) containing 500 mg/kg SS. Birds were coted with or without antibiotics. The growth experiment lasted for 10 wk. Results showed that birds fed the 50 mg/kg SS diet tended to have lower abdominal fat rate. The gene expression of liver X receptor- $\alpha$  (*LXR- $\alpha$* ) in liver and serum total cholesterol (TC) were dropped, and the gene expression of acyl-CoA

thioesterase 8 (*ACOT8*) in liver were upregulated. Compared with CON group, the levels of lysozyme, IL-10, and transforming growth factor (*TGF- $\beta$* ) in the serum were elevated as along with gene expression of *IL-10*, *TGF- $\beta$* , and *LYZ* in ileum of both 50 and 500 SS group. However, the level of secretory immunoglobulin A (*sIgA*) and *Mucin-2* in the ileum were downregulated in the 500 SS group. Additionally, *Lactobacillus* and *Lactobacillus gasseri* were the dominant bacteria in the 50 SS group, whereas the relative abundance of *Lactobacillus* was dropped in the 500 SS group. With combined antibiotics treatment, the  $\alpha$ -diversity of bacteria was reduced, and the biological effects of SS were eliminated. In conclusion, the lipid metabolism, immune function, and intestinal flora of the laying hens were improved with the dietary supplementation of 50 mg/kg SS. But dietary 500 mg/kg SS had negative effects on laying hens.

**Key words:** intestinal health, laying hen, lipid metabolism, soya saponin

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## INTRODUCTION

Soya saponin (SS) is composed of soybean sapogenin, some glycosides, uronic acid, or other organic acids. Some studies found that SS contributed to improving lipid metabolism and regulating immunity and soybean sapogenin or some glycosides might play an important role here (Guang et al., 2014). Past studies suggested that SS was difficult to be absorbed by intestinal epithelial cells and were generally degraded by intestinal microorganisms into soya saponol and then absorbed and utilized (Hu et al., 2004; Kamo et al., 2014). Based

on the difference in glycosides, SS is divided into 4 different types, that is, A, B, E, DDMP (Guang et al., 2014). Type A and B of SS are the most abundant in soybeans. At present, SS I in type B of SS is the most studied (Berhow et al., 2006).

Most of the in vivo studies on SS have been carried out on mice. A past study suggested that the proportion of Treg cells in mouse spleen and cochlear lymph nodes was significantly increased with 20 mg/kg SS supplementation (Nagano et al., 2019). The phosphorylation level of Y-box binding protein 1 (YB-1) was downregulated to inhibit the activation of NF- $\kappa$ B via adding SS (Wang et al., 2020). Some findings also suggested that SS relieved lung fibrosis by downregulating the expression of Fas and FasL (Nagano et al., 2017). Chen et al. found that SS alleviated the activation of NF- $\kappa$ B and the overexpression of *IL-1 $\beta$*  in mice liver induced by high-fat diet (Chen et al., 2014). While in poultry, antibody titer of Newcastle disease in broiler serum was

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elevated with 5 mg/kg SS oral treated (Naveed et al., 2020). Importantly, some inflammation models were constructed to study the biological functions of SS. The addition of 20 mg/kg SS in the diets alleviated the inflammation of lung and liver induced by lipopolysaccharide (LPS) in mice (Lin et al., 2016; Wang et al., 2020). The colitis caused by 3,4,5-trinitrobenzene sulfonic acid (TNBS) in mice was mitigated (Lee et al., 2010). Additionally, the disturbance of the intestinal microflora and intestinal inflammation induced by 2,4-dinitrofluorobenzene were also alleviated (Neacsu et al., 2020). Interestingly, the biological effects of SS were eliminated when vancomycin was used to treat the intestinal flora. It is well known that vancomycin mainly inhibits the growth of Gram-positive bacteria (Wang et al., 2011), which illustrate us that the biological effects of SS might be related to some Gram-positive bacteria.

These finding suggested that the biological effects of SS were closely related to intestinal bacteria. An example of this is that SS inhibited the proliferation of *Staphylococcus aureus* and *Escherichia coli* via inhibiting the activity of  $\beta$ -lactamase (Fussbroich et al., 2015; Horie et al., 2018). In another study, SS supplementation helped attenuate ear swelling and tissue edema caused by allergies via improving the intestinal flora and stimulating the proliferation of regulatory T cells, whereas it was blocked by vancomycin treatment (Nagano et al., 2019). In addition, a finding also suggested that the metabolized products of SS by the intestinal flora contained some neutral sterols and bile acids, and these metabolites could reduce serum cholesterol levels (Lee et al., 2005). Some studies attempted to ferment soybean meal with *Lactobacillus pentosus*, and finally soya saponol and genistein were detected (Yoo and Kim, 2015). Most of these research methods were outdated, and the results were not consistent. In addition, many studied could not find a direct connection between SS and intestinal flora. Although a number of studies indicated the role of SS in the regulation of immune function, relatively few studies explored its effect SS on poultry. Moreover, very limited information is available on the relationship between SS and intestinal flora in poultry.

It is generally accepted that vancomycin and neomycin show strong antibacterial effects on Gram-positive bacteria, and metronidazole has an inhibitory effect on Gram-negative bacteria. In addition, ampicillin has the ability to inhibit the proliferation of Gram-positive and Gram-negative bacteria (Wang et al., 2011). A combination of antibiotics (ampicillin, neomycin, metronidazole, and vancomycin) was used to disturb the intestinal bacteria and construct a low-intestinal bacteria model (Wang et al., 2011). We hypothesized that it could be interesting to explore the effect of SS under dysbacteriosis. Therefore, the above antibiotic combination was used to disturb the intestinal bacteria of laying hens, and the effects of SS on the intestinal bacteria, immune function, and lipid metabolism were studied with the intestinal bacteria disturbed or not.

## MATERIALS AND METHODS

All procedures adapted for the experiment were approved by the Animal Ethics Committee of China Agricultural University, Beijing, China. The animal welfare number was AW92601202-1-2.

### Experiment Design and Animal Management

This study was carried out at the Poultry Experiment Base of China Agricultural University (Zhuozhou, Hebei). A total of 540 Hy-line gray layer hens with 21-wk-old weights and similar egg production rates were selected and housed in a conventional stepped cage in a closed house. The cages were arranged in 3 tiers with 5 cages per tier and 3 birds per cage. One wk of prefeeding was carried out, and the control diet was fed during the pre-feeding. The diet formula of laying hens was formulated with reference to the feeding standards of Chinese chickens (NY/T33-2004) (Table 1). After the acclimation period, five hundred and forty 22-wk-old Hy-line gray hens were randomly divided into 6 treatment groups according to the principle of uniform egg production rate ( $47\% \pm 0.02\%$ ) and similar body weight ( $1470 \pm 10$  g). The dietary treatment were: control group (fed basal diet with low soybean meal), 50 ppm SS group (basal diet supplemented with 50 mg/kg SS), 500 ppm SS group (basal diet supplemented with 500 mg/kg SS), antibiotic group (K group; fed the diet of control group with 400 mg/kg ampicillin, 400 mg/kg neomycin, 400 mg/kg metronidazole, and 200 mg/kg vancomycin supplemented), K+ 50 SS group (fed the diet of K group with 50 mg/kg SS), and K+500 SS group (fed the diet of K

**Table 1.** Diet composition and nutrition level (air-dry basis).

Ingredients	Contents (%)	Nutritional parameters	Levels
Corn (7.8%)	67.55	ME MC/kg	2.70
Dephenolized cotton seed protein (50%)	14.00	Crude protein %	16.53
Limestone powder	8.154	Lysine%	0.79
Corn gluten meal (51.3%)	5.00	Methionine%	0.41
Soybean meal (48%)	2.00	Calcium %	3.63
Ca (HCO <sub>3</sub> ) <sub>2</sub>	1.86	Total phosphorus %	0.76
NaCl	0.35	Available phosphorus %	0.43
Trace minerals <sup>b</sup>	0.30	Methionine %	0.68
L-Lysine HCl (78%)	0.25	Threonine %	0.58
DL-Methionine	0.12	Tryptophan %	0.16
Choline chloride (50%)	0.12		
L-Tryptophan	0.02		
Multi-vitamins <sup>a</sup>	0.03		
Santoquin	0.03		
Phytase	0.016		
Zeolite powder	0.20		
Total	100		

<sup>a</sup>Vitamin premix (provided per kilogram of feed) the following substances: vitamin A, 12,500 IU; vitamin D3, 2,500 IU; vitamin K3, 2.65 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B12, 0.025 mg; vitamin E, 30 IU; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.

<sup>b</sup>Trace element premix (provided per kilogram of feed) the following substances: copper, 8 mg; zinc, 75 mg; iron, 80 mg; manganese, 100 mg; selenium, 0.15 mg; iodine, 0.35 mg.

group with 500 mg/kg SS). The antibiotics with purity greater than 98% were used in the experiment and were purchased from Wuhan Dongkang Technology Development Co., Ltd. There were 6 replicates per treatment and 15 birds per replicate. The test SS was purchased from Xi'an Tongze Biotechnology Co., Ltd. (total SS content was 45.1%). The dietary treatments were fed for 10 wk. The temperature of the laying hen room was controlled at  $25 \pm 3^\circ\text{C}$ , and a 16-h light:8-h dark lighting program was employed. At the end of the experiment period, all birds were weighed and 8 laying hens with uniform weight from each group were randomly selected to collect blood from the wing vein. Briefly, these birds were injected intravenously with 50 mg/kg BW of sodium pentobarbital (Zhao et al., 2017; Zhang et al., 2018), and quickly slaughtered after anesthesia to obtain abdominal fat, ileal tissue, chyme, and liver for subsequent testing. The proportion of abdominal fat was calculated according to the formula: abdominal fat percentage (%) = abdominal fat weight (g)/chicken live weight (g)  $\times$  100%.

### **Serum Biochemical Indicators, Immune Molecules, and Cytokines**

The birds were fasted for 8 h to collect blood from the wing vein, and then the blood was centrifuged at 3,000 rpm for 15 min at  $4^\circ\text{C}$ . The serum was separated and stored in a refrigerator at  $-80^\circ\text{C}$  for later use. An automatic biochemical analyzer (Unicel DXC800, Beckman Coulter Inc, Fullerton, CA) was used to determine the serum glucose (GLU) content. The kits from Nanjing Jiancheng Biotechnology Co., Ltd. were used to determine the levels of total cholesterol (TC), triglycerides (TG), lysozyme (LYZ) and complement C3 in the serum. A chicken  $\beta$ -defensin 1 enzyme-linked immunoassay kit from Beijing Konka Hongyuan Biotechnology Co., Ltd. was used to detect the content of  $\beta$ -defensin in the serum. The serum levels of TNF- $\alpha$ , IL-10, IL-1 $\beta$ , and TGF- $\beta$  were tested according to the method of ELISA kit (IDEXX Laboratories Inc., Westbrook, ME).

### **The sIgA Level of Ileum Mucosa**

After the ileal mucosa samples of laying hens were collected, the tissue homogenate was prepared according to the ratio of mucosal sample: normal saline = 1:9, and then centrifuged at 3000 rpm and  $4^\circ\text{C}$  for 15 min to separate the supernatant for use. The chicken secretory sIgA ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) was used to determine the content of sIgA. The total protein level was measured according to the protocol of the BCA protein quantification kit (Cwbio, Beijing, China). The value of sIgA was expressed as the level of sIgA per gram of protein.

### **Intestinal Morphology**

The middle ileum of laying hens was collected about 1 cm in length and suspended in 4% paraformaldehyde

solution, tissue sections were prepared and stained with periodic acid Schiff stain (PAS staining). With reference to the method of Wagner et al. (1999), relevant data such as the height of the ileum villi and the depth of the crypts of the laying hens were measured. Briefly, for each intestinal section, 10 longest, straight and intact intestinal villi were selected for measurement. The Olympus BX-41TF microscope and a thousand-screen high-definition color pathology graphic analysis system were used to measure the height of the ileal mucosal villi and the depth of the crypts. The ratio of the height of the villi to the depth of the crypts was calculated. Additionally, the goblet cells within 100 microns of each intestinal villi were counted to count their number.

### **Gene Expression**

Liver and ileum were collected and placed in RNase-free Centrifuge tube, and then the samples were quickly placed in liquid nitrogen. 100 mg tissue sample was put into a 1mL trizol (Invitrogen Life Technologies, Carlsbad, CA), Total RNA isolation was carried out as previously described by Fan et al. (2018). Briefly, after the total RNA was quantified and the purity was assessed, the integrity of RNA in each sample was assessed using 1% denatured agarose gel electrophoresis. Total RNA was reverse-transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instruction. The RT-PCR analysis of gene expression was performed using primers listed in Table 2, and the SYBR Premix Ex TaqTM (Takara, Dalian, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA). The total volume of the PCR reaction system was 20  $\mu\text{L}$ . Amplification products were verified by melting curves, agarose gel electrophoresis, and direct sequencing. Results were analyzed by the cycle threshold (CT) method from Fu et al. (2010).

### **16srDNA Sequencing of Ileal Bacteria**

The chyme in the distal region of the ileum was collected at end of the 10th wk. Sequencing and analysis according to the method described by Zhang et al. (2018). Briefly, the fecal microbial DNA extraction kit (QIAamp Fast DNA Stool Mini Kit, Qia-gen Company, Dusseldorf, Germany) was used to extract microbial DNA. After the concentration of the DNA sample was determined, 1% agarose gel electrophoresis was used to detect the purity of DNA samples. The universal primers of 16SrDNA gene V3-V4 region were used to amplify bacterial DNA, the specific primer sequence numbers are as follows, 338 F (5'-ACTCCTACGGGAGG-CAGCA-3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3'). After that, the PCR products were purified, quantified and homogenized to form a sequencing library. HiSeq2500 PE250 was used for on-machine sequencing. Sequencing analysis was completed by Beijing Nuohe Zhiyuan Bio-Information

**Table 2.** List of gene primer sequences<sup>a</sup>.

Gene name <sup>b</sup>	Prime sequence (5'-3')	NCBI number
<i>ACC</i>	F-AATGGCAGCTTTGGAGGTGT R-TCTGTTTGGGTGGGAGGTG	XM_015295697.3
<i>LxR<math>\alpha</math></i>	F-CAAAGGGAATGAATGAGC R-AGCCGAAGGGCAAACAC	XM_040700552.1
<i>SREBF1</i>	F-GCCCTCTGTGCCTTTGTCTTC R-ACTCAGCCATGATGCTTCTTC	XM_015294109.3
<i>ACOT8</i>	F-CCACTCGCTTCACTGCTACTTCG R-TGGCACATCTTCAGCTTGGATCTTG	XM_040688278.1
<i>PPAR-<math>\delta</math></i>	F-TGAATGACCAAGTGACTCTGCTGAAG R-CAGTGCTCGGAGGATGTTGTCTTG	XM_040652685.1
<i>PPAR-<math>\alpha</math></i>	F-TCCCTTCCCCTGACCAAAA R-TCCTGCACTGCCTCCACA	NM_001001460.1
<i>TNF-<math>\alpha</math></i>	F-GAGCGTTGACTTGGCTGTC R-AAGCAACAACCAGCTATGCAC	XM_040647309.1
<i>IL-1<math>\beta</math></i>	F-ACTGGGCATCAAGGGCTA R-GGTAGAAGATGAAGCGGGTCT	XM_015297469.2
<i>TGF-<math>\beta</math></i>	F-TCATCACCAGGACAGCGTTA R-TGTGATGGAGCCATTTCATGT	NM_001031045.3
<i>Mucin-2</i>	F-TCCCTGTTGAGGGAGAACTT R-AGTGGTTGTACCTTCGGTGC	XM_040673077.1
<i>Claudin-1</i>	F-AAAGTGCATGGAGGATGACCA R-GCCACTCTGTTGCCATACCA	NM_001013611.2
<i>ZO-1</i>	F-TATGAAGATCGTGCGCCTCC R-GAGGTCTGCCATCGTAGCTC	XM_040680632.1
<i>IL-10</i>	F-CGCTGTCACCGCTTCTTCA R-CGTCTCCTTGATCTGCTTGATG	NM_001004414.2
<i>LYZ</i>	F-CCCAGGCTCCAGGAACCT R-CACGCTCGCTGTTATGTCTGA	NM_205281.1
<i>MHC-II</i>	F-ATAAGCGTGGGCTCAGTTC R-GAATTCGGGCAGCCTCCATA	NM_001245061.1
<i><math>\beta</math>-actin</i>	F-GAGAAATTGTGCGTGACATCA R-CCTGAACCTCTCATTGCCA	NM_205518.1

<sup>a</sup>Primers designed using Primer Express software (Sangon Biotech, Shanghai, China).

<sup>b</sup>Abbreviations: *ACC*, acetyl coenzyme A dehydrogenase; *ACOT8*, acyl-CoA thioesterase 8; *Claudin-1* and *ZO-1* belong to tight junction proteins; *IL-1 $\beta$*  and *IL-10*, interleukin 1 $\beta$  and 10; *LxR $\alpha$* , liver X receptor- $\alpha$ ; *LYZ*, lysozyme; *MHC-II*, major histocompatibility complex II; *Mucin-2*, mucin family protein 2; *PPAR- $\delta$*  and *PPAR- $\alpha$* , peroxisome proliferators-activated receptor- $\delta$  and  $\alpha$ ; *SREBF1*, sterol regulatory element binding transcription factor 1; *TGF- $\beta$* , transforming growth factor  $\beta$ ; *TNF- $\alpha$* , tumor necrosis factor  $\alpha$ .

Technology Co., Ltd. Qiime software (Qiime2-2019.7, Nature Biotechnology) was used to generate species abundance tables of different taxonomic levels. The alpha diversity of the samples was analyzed, at the same time the UPGMA clustering tree was constructed. Additionally, LEfSe analysis was performed to find biomarkers with statistical differences between the groups based on the LDA value. R software (Version 2.15.3) was used to draw principal coordinate analysis (**PCoA**) diagram.

### Statistical Analysis

The conventional linear model program in SPSS 23.0 software (SPSS Inc., Chicago, IL) was used to analyze the data. Two-way ANOVA was performed in a 2  $\times$  3 factor arrangement to analyze the effects of combined antibiotic treatment and SS, as well as the interaction of these 2 factors, among which the effects of SS were compared by Duncan's multiple comparisons. When the interaction of combination antibiotics and SS was observed, One-way ANOVA and Duncan's multiple comparisons were used. The Pearson correlation coefficient was used to reflect the correlation between the immune-related indicators and the bacteria with differences in this study.  $P < 0.05$  was considered to be significant and  $0.05 < P < 0.10$  was considered to have a trend of difference. Graphpad prism 8.0 software was used to graph the data.

## RESULT

### Lipid Metabolism

Compared with the control group, the abdominal fat rate of laying hens in the 50 SS group tended to be decreased ( $P = 0.057$ ), and the level of total cholesterol (TC) in the serum was reduced ( $P < 0.05$ ) (Table 3). The body weight of laying hens was not affected by 50 mg/kg SS and antibiotic treatment. Whereas dietary 500 mg/kg SS increased the levels of serum TC, triglycerides (TG), and body weight at the end of the trial. The results of genes in the liver showed the mRNA level of *ACOT8* was upregulated, and the transcription level of *LxR- $\alpha$*  was downregulated in the 50 SS group compared with the control group ( $P < 0.05$ ) (Figure 1). The above results inspired us that dietary 50 mg/kg SS might improve the fat metabolism of laying hens via elevating liver fatty acid  $\beta$ -oxidation and downregulating fatty acid synthesis.

### Immune and Intestinal Barrier Function

With antibiotic treated, the levels of serum TGF- $\beta$  and sIgA in the ileal mucosa were descended (Table 4, and Figure 2B). In addition, the distribution of ileum villi in layers treated with antibiotics was

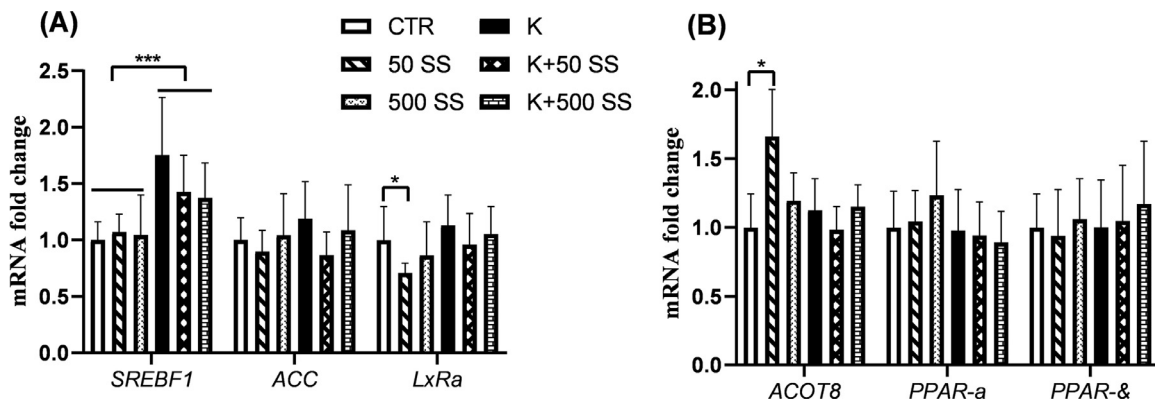
**Table 3.** Results of body weight, abdominal fat ratio and serum biochemical indicators.

Antibiotic <sup>1</sup>	SS, mg/kg	Initial weight, kg	End weight, kg	Abdominal fat, %	TC, mmol/L	TG, mmol/L	GLU <sup>2</sup> , mmol/L
(-)	0	1.470	1.496	3.39	5.58 <sup>c</sup>	1.53 <sup>a</sup>	12.15
	50	1.472	1.488	2.71	4.85 <sup>a</sup>	1.57 <sup>a</sup>	12.23
	500	1.472	1.513	3.12	5.4 <sup>bc</sup>	2.33 <sup>b</sup>	12.19
(+) )	0	1.471	1.475	2.72	5.09 <sup>ab</sup>	2.39 <sup>b</sup>	12.25
	50	1.472	1.505	2.53	5.27 <sup>abc</sup>	2.41 <sup>b</sup>	11.78
	500	1.472	1.536	3.40	5.65 <sup>c</sup>	2.43 <sup>b</sup>	11.79
SEM		0.002	0.005	0.091	0.058	0.041	0.072
Main effects							
Antibiotic	(-)	1.472	1.499	3.07	5.28	1.81 <sup>a</sup>	12.19
	(+)	1.471	1.505	2.88	5.34	2.41 <sup>b</sup>	11.94
SS	0	1.470	1.485 <sup>a</sup>	3.054 <sup>ab</sup>	5.34 <sup>ab</sup>	1.96 <sup>a</sup>	12.20
	50	1.472	1.496 <sup>a</sup>	2.620 <sup>a</sup>	5.06 <sup>a</sup>	1.99 <sup>a</sup>	12.00
	500	1.472	1.524 <sup>b</sup>	3.262 <sup>b</sup>	5.52 <sup>b</sup>	2.38 <sup>b</sup>	11.99
Main effects and interactions							
Antibiotic		0.989	0.556	0.301	0.603	<0.001	0.087
SS		0.935	0.014	0.019	0.009	<0.001	0.426
Antibiotic*SS		0.988	0.196	0.113	0.006	<0.001	0.238

<sup>a,b,c</sup>Means in the same column without common superscripts differ significantly ( $P < 0.05$ ).

<sup>1</sup>(-) Means not added, (+) means to add.

<sup>2</sup>GLU, glucose.



**Figure 1.** The effect of SS on liver genes transcription level. Among them, \* represents  $0.01 < P < 0.05$ , and \*\*\* represents  $P < 0.001$ . CTR = control group, 50 SS= 50 mg/kg SS group, 500 SS= 500 mg/kg SS group, K = antibiotic group, K+50SS = antibiotic with 50 mg/kg SS supplemented group, K+500 SS = antibiotic with 500 mg/kg SS supplemented group, the same below.

uneven, and the height of villi was shorter than that in the groups without antibiotics treated (Figure 3). Additionally, the number of goblet cells in the ileum was reduced as well as the transcription level of *Mucin-2*, *IL-*

*1β*, and *MHC-II* in the ileum (Figure 2A, C and Figure 4). The levels of serum lysozyme, IL-10, and TGF- $\beta$  were elevated with 50 and 500 mg/kg SS addition ( $P < 0.05$ ) (Table 4). Additionally, the gene mRNA

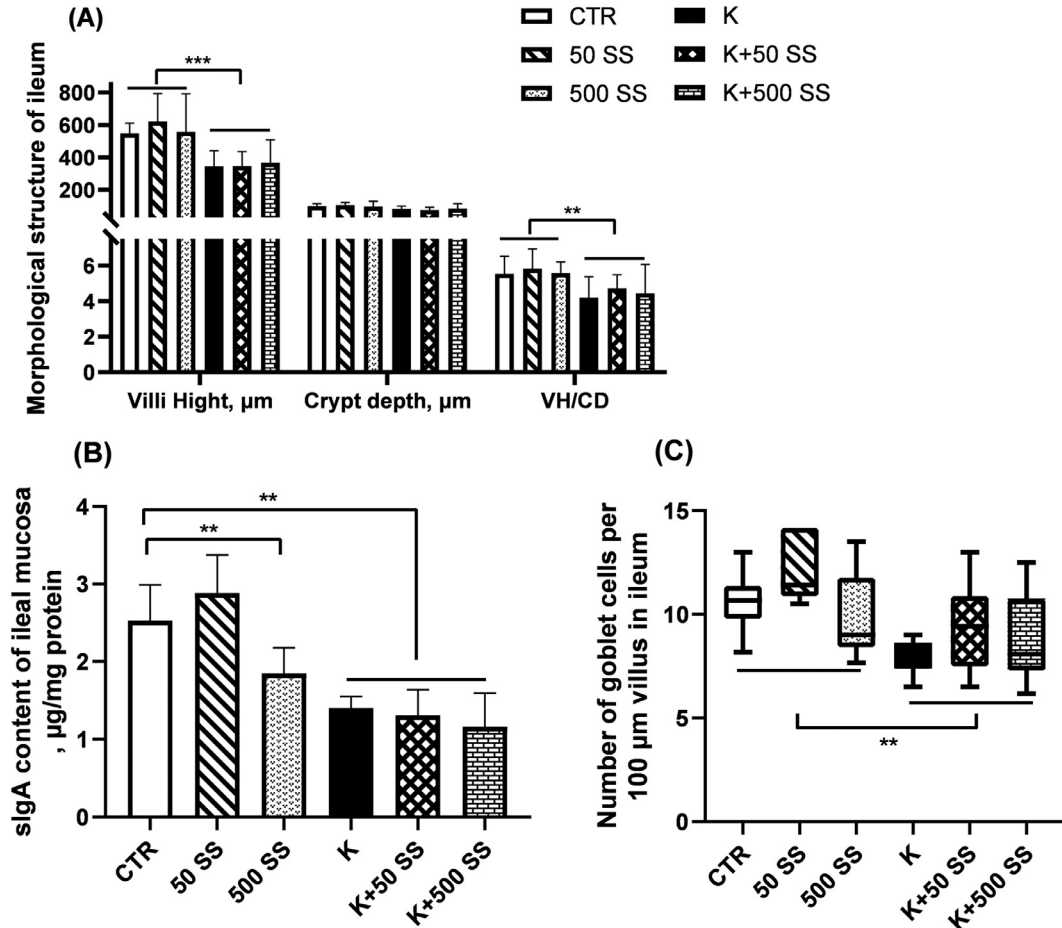
**Table 4.** The levels of serum immune molecules and cytokines.

Antibiotic <sup>1</sup>	SS, mg/kg	IL-1 $\beta$ , pg/mL	IL-10, pg/mL	TNF- $\alpha$ , pg/mL	TGF- $\beta$ , pg/mL	C3 <sup>2</sup> , g/L	Lysozyme, U/mL	$\beta$ -defensins, ng/L
(-)	0	19.06	21.10	115.91 <sup>b</sup>	31.72	0.59	126.79	7.32
	50	18.58	26.36	103.26 <sup>ab</sup>	35.59	0.60	164.63	7.06
	500	18.25	25.52	107.88 <sup>ab</sup>	36.05	0.54	159.52	7.18
(+) )	0	18.81	21.22	99.42 <sup>a</sup>	26.90	0.55	148.34	7.61
	50	19.27	24.29	106.26 <sup>ab</sup>	33.61	0.65	145.81	7.47
	500	21.12	23.93	109.22 <sup>ab</sup>	34.92	0.62	152.81	7.71
SEM		0.357	0.490	1.708	0.748	0.013	3.355	0.228
Main effect								
Antibiotic	(-)	18.63	24.32	109.02	34.45	0.58	150.32	7.19
	(+)	19.73	23.15	104.97	31.81	0.61	148.99	7.60
SS	0	18.94	21.16 <sup>a</sup>	107.66	29.31 <sup>a</sup>	0.57	137.57 <sup>a</sup>	7.47
	50	18.93	25.32 <sup>b</sup>	104.76	34.60 <sup>b</sup>	0.63	155.22 <sup>b</sup>	7.27
	500	19.68	24.72 <sup>b</sup>	108.55	35.49 <sup>b</sup>	0.58	156.17 <sup>b</sup>	7.45
Main effects and interactions								
Antibiotic		0.130	0.236	0.243	0.085	0.303	0.844	0.374
SS		0.613	0.002	0.642	0.003	0.186	0.049	0.924
Antibiotic*SS		0.198	0.636	0.045	0.579	0.167	0.052	0.976

<sup>a,b</sup>Means in the same column without common superscripts differ significantly ( $P < 0.05$ ).

<sup>1</sup>(-) Means not added, (+) means to add.

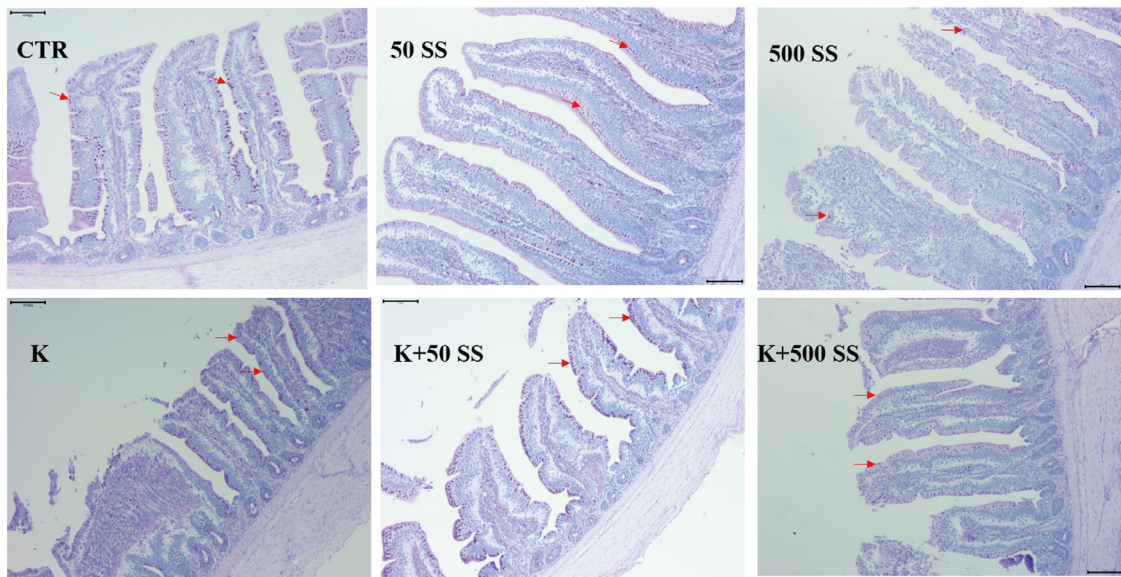
<sup>2</sup>C3, complement C3.



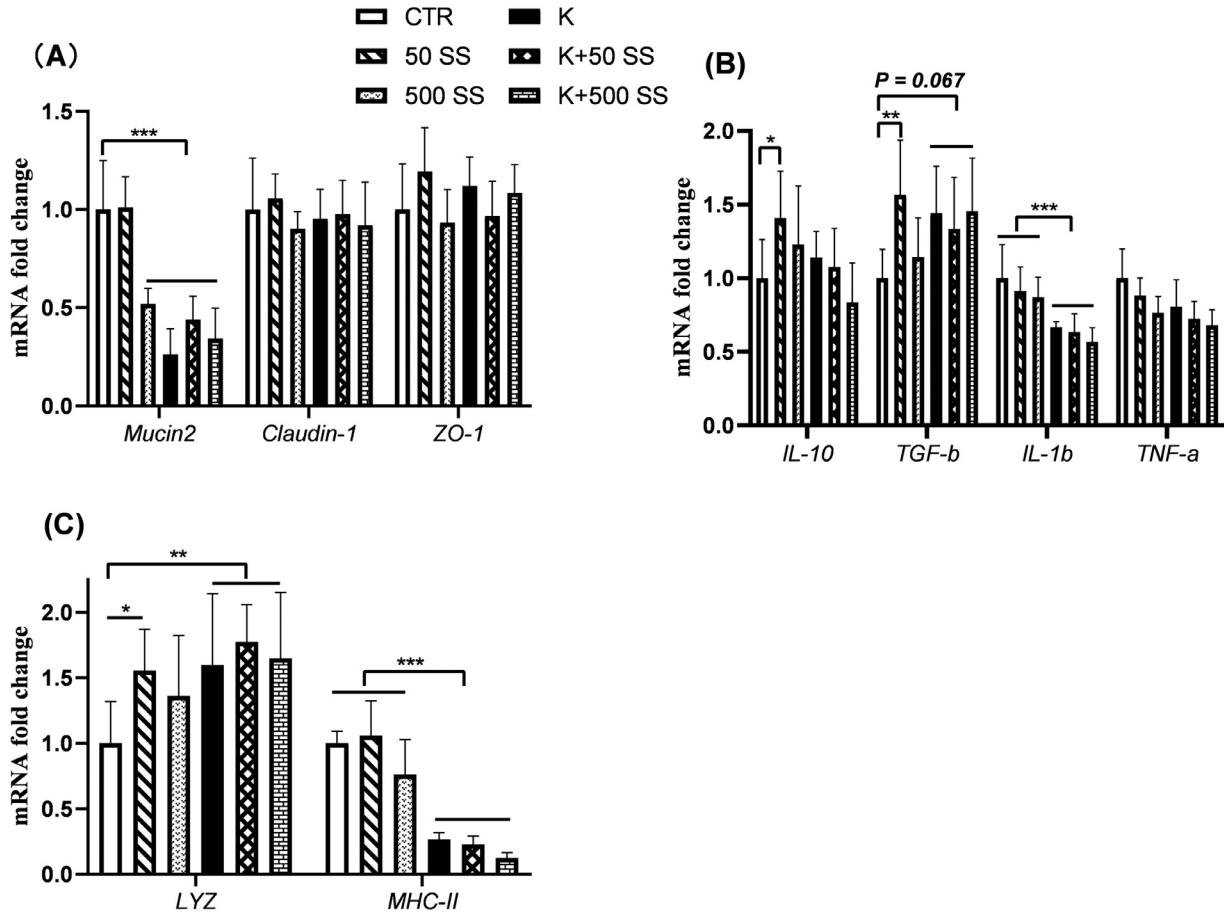
**Figure 2.** The effect of SS on ileum morphology and sIgA content of ileal mucosa. The ileum morphology and goblet cell structure are shown in (A) and (C), respectively. The sIgA level is shown in (B). Among them, \*\* represents  $0.001 < P < 0.01$ , and \*\*\* represents  $P < 0.001$ .

levels such as *IL-10*, *TGF- $\beta$* , and *LYZ* in the ileum were upregulated in the 50 SS group compared with the control group ( $P < 0.05$ ) (Figure 4B, C). While dietary 500 mg/kg SS dropped the level of sIgA in the ileal

mucosa (Figure 2B), and the mRNA level of *Mucin-2* in the ileum (Figure 4A). Our results illuminated us that dietary 50 and 500 mg/kg SS might be beneficial to the innate immune function of laying hens, whereas the



**Figure 3.** The intuitive diagram of ileum morphology and goblet cells. The goblet cells are marked by red arrows. CTR = control group, 50 SS = 50 mg/kg SS group, 500 SS = 500 mg/kg SS group, K = antibiotic group, K+50SS = antibiotic with 50 mg/kg SS supplemented group, K+500 SS = antibiotic with 500 mg/kg SS supplemented group, the same below.



**Figure 4.** The effect of SS on ileum gene transcription level. Among them, \* represents  $0.01 < P < 0.05$ , \*\* represents  $P < 0.01$ , and \*\*\* represents  $P < 0.001$ .

intestine mucosal immune function seemed to be negatively affected with 500 mg/kg SS treated. It might be related to the changes in the intestinal flora. Additionally, the intestinal barrier and immune function of laying hens were negatively affected with antibiotic treated. It was worth mentioning that with a combination of antibiotics treated, the biological effects of SS were eliminated. To gain more insight, the structure of the intestinal flora was analyzed.

### Ileal Flora

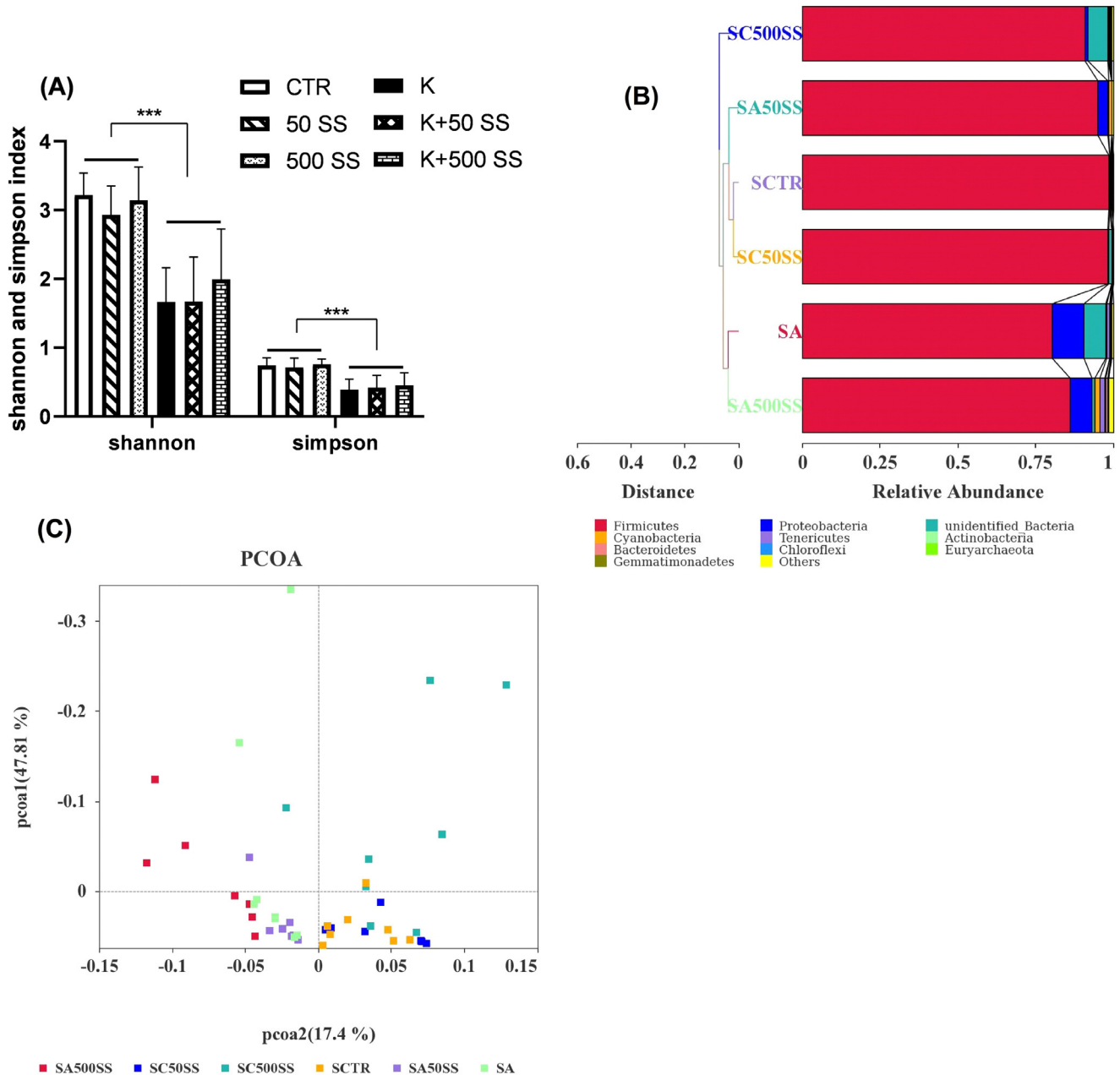
With antibiotic treated, the alpha diversity of the ileal flora was reduced (Figure 5A), and the beta diversity of the ileal flora was different in antibiotic treatment or not (Figure 5C). LEfSe analysis showed the *Proteobacteria*, *Helicobacter*, and *Escherichia coli* were the dominant bacteria with antibiotic treated (Figure 6). In addition, the relative abundance of *Firmicutes*, *Romboutsia*, *Lactobacillus-pontis*, and *Lactobacillus-gasseri* were dropped. At the same time, the relative abundance of the *Proteobacteria*, *Helicobacter*, and *Lactobacillus salivarius* were elevated ( $P < 0.05$ ) (Figure 7A–F). The structure about the ileal bacteria of the 50 SS group was similar to that of the control group (Figure 5B). Interestingly, the beneficial bacteria such as *Lactobacillus* and *Lactobacillus gasseri* in the ileal

chyme of the 50 SS group were the dominant bacteria, and the relative abundance of *Lactobacillus-pontis* was raised ( $P < 0.05$ ) (Figures 6 and 7F). In our study, the structure about the ileal bacteria both the 500 SS group and the control group were different (Figure 5B). Compared with the control group, the relative abundance of *Romboutsia* and *Lactobacillus-pontis* of the ileal bacteria were elevated in the 500 SS group (Figure 7D, F). Unfortunately, the relative abundance of *Lactobacillus* was reduced ( $P < 0.05$ ) (Figure 7D). Dietary 50 and 500 mg/kg SS alleviated the increase in the relative abundance of *Helicobacter* caused by the combined antibiotic treatment ( $P < 0.05$ ) (Figure 7D).

Our results indicated that combination of antibiotics could build a low-bacterial model in the intestine. The structure of the intestinal flora was improved with 50 mg/kg SS supplementation. In order to deeply investigate the relationship between the intestinal flora and the host, the correlation analysis was carried out.

### Correlation Analysis

The results of correlation analysis showed that *Firmicutes* and *Lactobacillus* showed a negative correlation with the level of serum TG ( $P < 0.05$ ) (Table 5). The relative abundance of *Lactobacillus gasseri* and *Lactobacillus pontis* showed a positive correlation with the mRNA



**Figure 5.** The structure of the flora in the ileum chyme. The  $\alpha$ -diversity and  $\beta$ -diversity are shown in (A) and (C), respectively. The result of the bacterial clustering tree is shown in (B). Among them, \*\*\* represents  $P < 0.001$ .

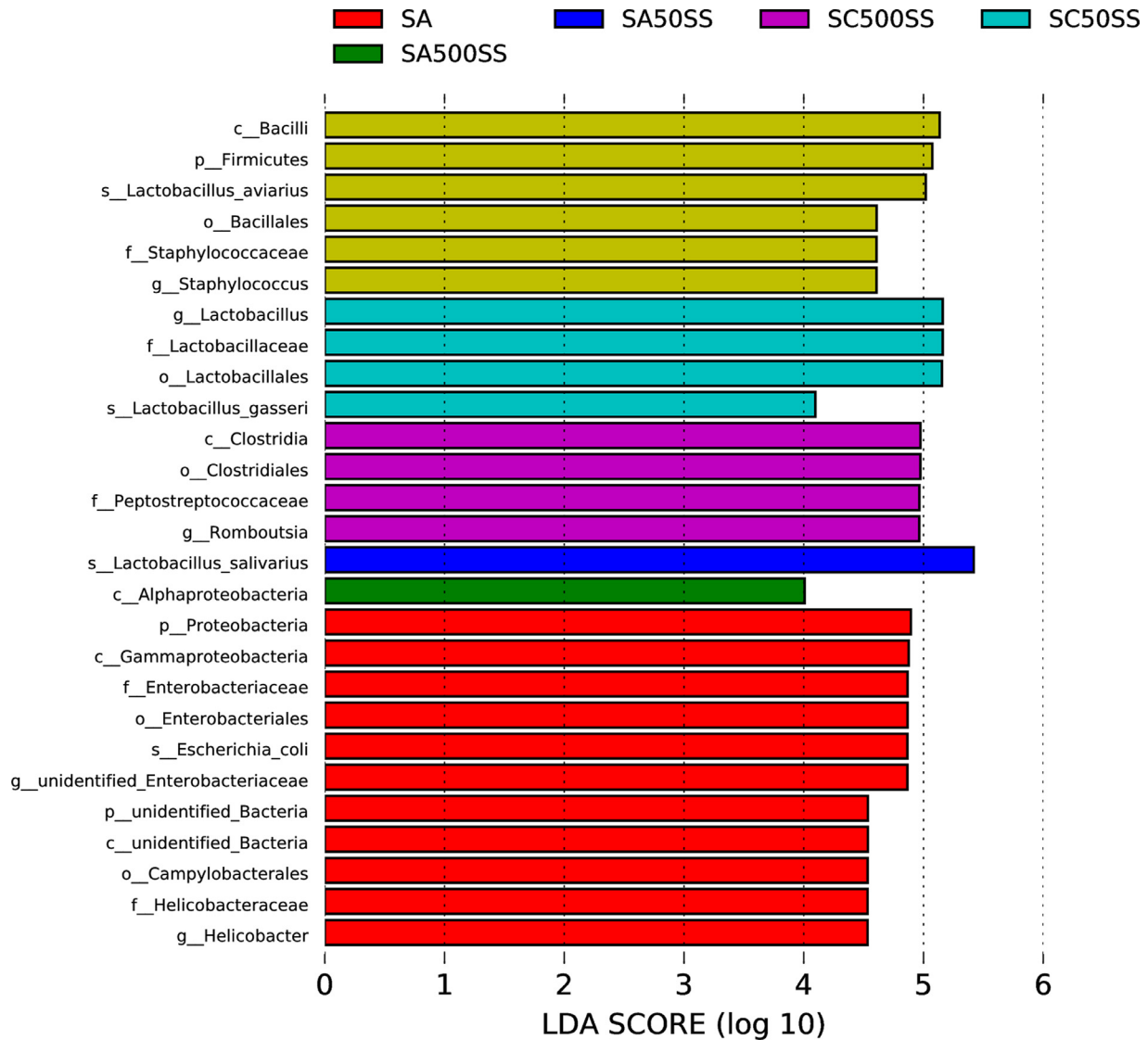
levels of *ACOT8* in the liver. Additionally, *Lactobacillus gasseri* showed a negative correlation with the level of serum TG and the mRNA levels of *LxR- $\alpha$*  in the liver ( $P < 0.05$ ). In this study, we also found the relative abundance of *Lactobacillus gasseri*, *Lactobacillus pontis*, and *Lactobacillus reuteri* showed a positive correlation with the mRNA levels of *Mucin 2* and *MHC-II* in ileum ( $P < 0.05$ ) (Figure 8). While the relative abundance of *Escherichia coli* showed a negative correlation with them. We also found that the relative abundance of *Romboutsia* showed a positive correlation with the serum TGF- $\beta$  and the mRNA levels of *MHC-II* in ileum (Figure 7). This indicated that dietary 50 mg/kg SS might improve lipid metabolism and immune function via optimizing the structure of intestinal flora. Whereas the relative abundances of those bacteria were dropped

with the combination of antibiotics treated, hence the biological effects of SS were eliminated.

## DISCUSSION

SS is a biologically active substance in legumes that improves lipid metabolism and regulates immune function. It has been suggested that dietary supplementation of 20 mg/kg SS improved the secretion of bile acids in mice, and the levels of serum cholesterol and high-density lipoprotein (HDL) were dropped (Lee et al., 2005). Also, with 20 mg/kg SS supplementation in high-fat diet, the levels of total cholesterol (TC) and triglycerides (TG) in the serum were decreased, and the synthesis of liver lipids was also inhibited (Xie et al., 2018). In



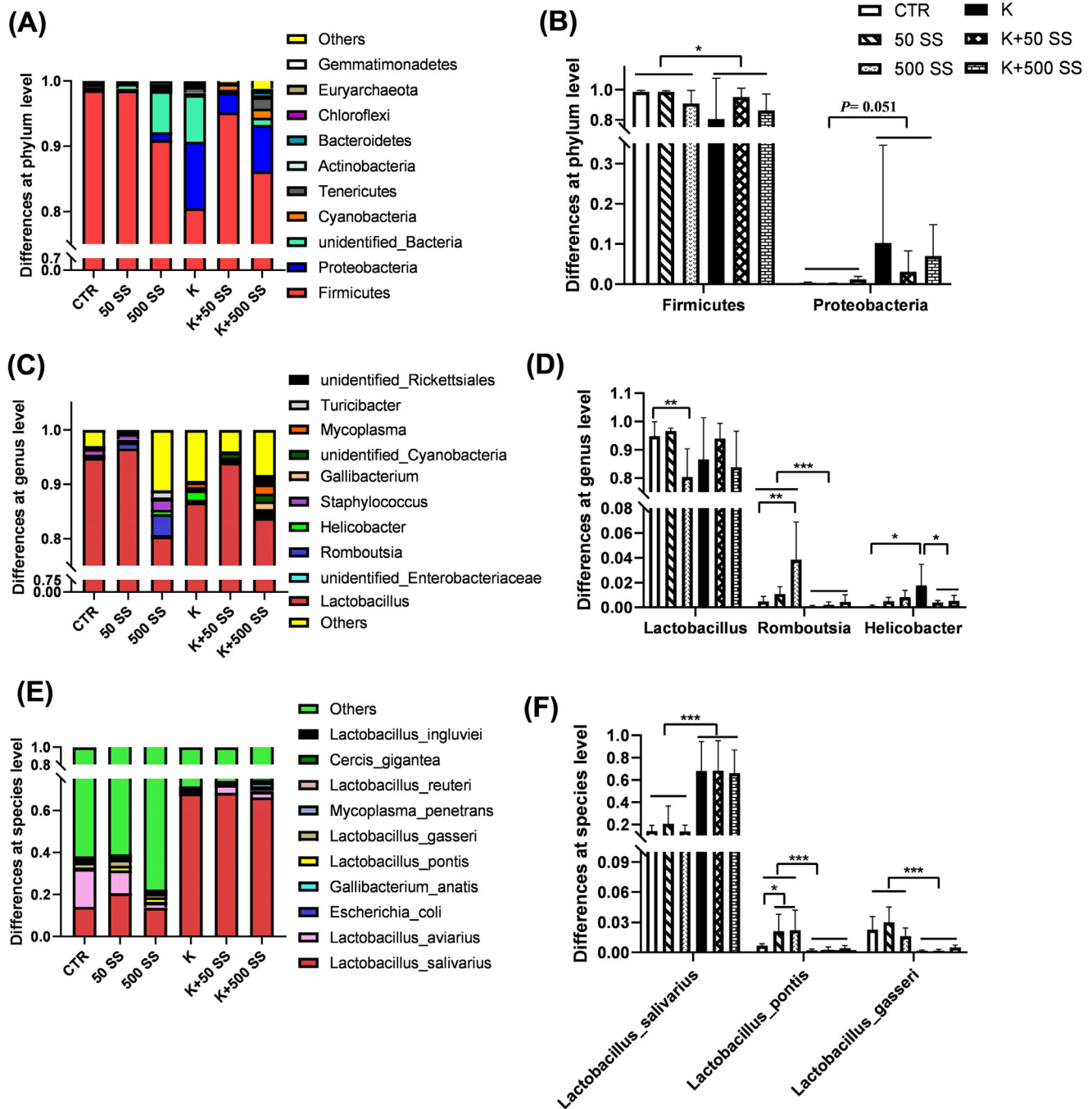


**Figure 6.** The LEfSe analysis results of ileal chyme bacteria. SCTR = control group, SC50SS = 50 mg/kg SS group, SC500SS = 500 mg/kg SS group, SA = antibiotic group, SA50SS = antibiotic with 50 mg/kg SS supplemented group, SA500SS = antibiotic with 500 mg/kg SS supplemented group, the same below.

the present study, the abdominal fat rate and the level of serum TC were decreased, and there was no negative effect on the body weight with 50 mg/kg SS supplementation, which was consistent with many past studies (Lee et al., 2005; Xie et al., 2018). The liver is the main site of lipid metabolism in the body. Thus, we quantified the mRNA levels of genes related to liver lipid metabolism. We found that the mRNA level of acyl-CoA thioesterase 8 (*ACOT8*) in the liver was upregulated, and the mRNA level of the liver X receptor- $\alpha$  (*LXR- $\alpha$* ) was downregulated in 50 SS group. Some studies suggested that *LXR- $\alpha$*  was a lipid regulator in the liver, which could establish a positive feedback loop with *SREBP-1c* and participated in the regulation of lipid synthesis in the liver (Willy et al., 1995). *LXR- $\alpha$*  also formed a heterodimer with the retinoic acid X receptor (RXR), which regulated the cholesterol and fatty acid homeostasis via regulating the expression of various enzymes and transporters involved in the lipid metabolism process (Hong and Tontonoz, 2014). *ACOT8* acted as an

auxiliary enzyme in the  $\beta$ -oxidation of various lipids to promote the  $\beta$ -oxidation of lipids (Hunt et al., 2012). *ACOT8* was activated under the induction of Peroxisome proliferator activated receptor family proteins to further terminate fatty acid synthesis (Hunt and Alexson, 2002). Based on the present findings, it should be noted that SS might improve the fat metabolism of laying hens via elevating liver fatty acid  $\beta$ -oxidation and downregulating fatty acid synthesis.

The regulatory effect of SS on immune function has been confirmed in a large number of in vitro and in vivo studies. The levels of serum lysozyme, defensins, and complement C3 were used to evaluate the strength of the innate immune response (Kim et al., 2012). In the present study, the level of serum lysozyme and the mRNA level of *LYZ* in the ileum were raised with 50 and 500 mg/kg SS supplementation. It indicated that SS might help the innate immunity of laying hens. The level of serum cytokines was used as another important indicator for evaluating the innate immunity (Siebert and



**Figure 7.** The effect of SS on the relative abundance of bacteria in the ileal chyme. The relative abundance of bacteria at the phylum level was shown in (A) and (B). The relative abundance of bacteria at the genus level was shown in (C) and (D), and the relative abundance of bacteria at the species level was shown in (E) and (F). Among them, \* represents  $0.01 < P < 0.05$ , \*\* represents  $P < 0.01$ , and \*\*\* represents  $P < 0.001$ .

Walker, 2010). Cytokines are divided into pro-inflammatory factors (such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6, etc.) and anti-inflammatory factors (for instance IL-10, TGF- $\beta$ , and IL-4, etc.). The appropriate expression of proinflammatory factors activates the immune system of the body, and once the expression is increased, it will cause an inflammatory reaction (Smith and Humphries, 2009). Anti-inflammatory factors are involved in immune tolerance and antibody synthesis; however, high expression levels also lead the body to disease susceptibility (O'Garra and Vieira, 2007). The coordinated expression of these cytokines maintains the body's immune homeostasis. In the present study, with 50 and 500 mg/kg SS

supplementation, the levels of IL-10 and TGF- $\beta$  in serum were elevated. At the same time, the mRNA levels of IL-10 and TGF- $\beta$  in the ileum were also upregulated. It illuminated us that dietary 50 and 500 mg/kg SS helped the body's immune homeostasis to shift towards the anti-inflammatory direction. To explain the observed activity, we might consider that SS stimulated the proliferation of immune cells and regulated the immune response due to its complex glycosyl side chain structure and small molecular structural units.

It is generally accepted that an integral intestinal morphology is the basis for evaluating intestinal health. In this study, we did not find that 2 dietary levels of SS

**Table 5.** Results of the correlation analysis between intestinal flora and lipid metabolism indexes

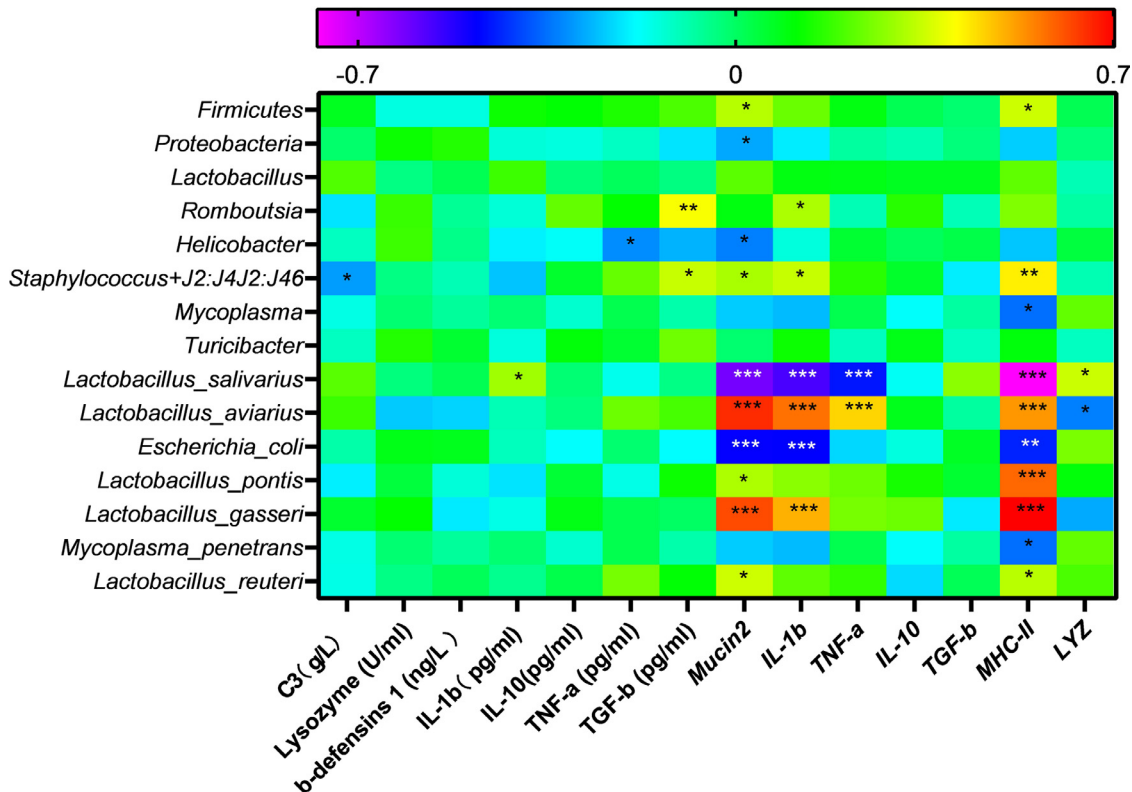
	Abdominal fat rate	TC	TG	<i>LxRa</i>	<i>ACOT8</i>
<i>Firmicutes</i>	0.141	-0.022	-0.318*	-0.177	0.070
<i>Proteobacteria</i>	-0.247	0.035	0.184	0.125	-0.108
<i>Lactobacillus</i>	-0.020	-0.105	-0.352*	-0.178	0.018
<i>Romboutsia</i>	0.089	-0.017	0.034	-0.174	0.097
<i>Helicobacter</i>	-0.143	-0.243	0.266	0.161	0.055
<i>Lactobacillus_salivarius</i>	-0.069	-0.011	0.517***	0.249	-0.187
<i>Lactobacillus_pontis</i>	-0.069	-0.238	-0.189	-0.226	0.329*
<i>Lactobacillus_gasseri</i>	0.177	-0.183	-0.639***	-0.395**	0.344*
<i>Lactobacillus_reuteri</i>	0.129	0.150	-0.399**	-0.055	0.389**

The Pearson correlation coefficient between intestinal flora and lipid metabolism indexes are shown in Table 5. Among them, \*Represents  $0.01 < P < 0.05$ . \*\*Represents  $P < 0.01$ . \*\*\*Represents  $P < 0.001$ .

had a negative effect on the ileum morphology and structure of laying hens. The secretion of sIgA is regulated by the number and types of flora in the intestinal lumen. At the same time, sIgA prevents pathogens in the intestinal lumen from colonizing the intestinal mucosa. It is regarded as the main immune barrier that maintains the homeostasis of the symbiotic flora (Papp et al., 2013). Mucins secreted by goblet cells cover the intestinal mucosa to form a thick mucus layer. Intestinal symbiotic bacteria generally colonize the outer mucus layer. The polymerized Mucin2 network structure prevents intestinal bacteria from invading the mucosal layer (Amiri et al., 2021). In the present study, one unanticipated finding was that the sIgA level of the ileal mucosa and the mRNA level of the *Mucin-2* in the ileum were dropped with 500 mg/kg SS supplementation, whereas

the number of goblet cells was not affected by the addition of SS. It was revealed that high doses of SS might have a negative impact on the intestinal mucosal immunity. We hold that the negative effects of high-dose SS on the intestinal mucosa might be related to the intestinal flora. To further explore this concept, the structure of the intestinal flora was analyzed.

The immune response and barrier function of the intestinal mucosa are regulated by the structure of the intestinal bacteria. Generally, SCFAs and functional oligosaccharides are metabolized by *Lactobacillus* bacteria to improve intestinal health, whereas the endotoxins produced by some harmful bacteria such as pathogenic *Escherichia coli*, *Campylobacter jejuni*, *Clostridium perfringens* damage intestinal health (Lin et al., 2017; Hiippala et al., 2018). In our study, we observed a



**Figure 8.** The results of the correlation analysis between the immune-related indicators and the bacteria with differences in this study. Among them, \* represents  $0.01 < P < 0.05$ , \*\* represents  $P < 0.01$ , and \*\*\* represents  $P < 0.001$ .

decrease in the relative abundance of *Lactobacillus* and an increase in the relative abundance of *E. coli* in the 500 SS group unexpectedly. In addition, *Lactobacillus* showed a negative correlation with the level of serum TG and the mRNA levels of *LxR- $\alpha$*  in the liver. The abdominal fat level was increased with high-dose SS treated, which might be related to the intestinal flora. It demonstrated that high dose of SS had a negative impact on the ileal bacteria population. A possible explanation was that the high-dose of SS had a strong ability to stimulate the intestinal mucosal immunity. Therefore, it could be speculated that harmful bacteria were more adaptable to the environment evolved into the dominant intestinal bacteria. It seemed plausible that ileum mucosal immune function was weakened by the addition of high-dose of SS. Our previous study found high-dose SS had a negative impact on egg-laying performance (Li et al., 2021), which might be related to the changes in intestinal flora caused by high-dose SS in this study.

It has been suggested that *Lactobacillus gasseri* activated TLR2/6 and promoted the proliferation of lymphocytes (Stoeker et al., 2011), thereby the levels of IL-12 and IL-10 were elevated. It alleviated colitis in IL-10 deficient mice (Carroll et al., 2007; Luongo et al., 2013). Similarly, *Lactobacillus gasseri* regulated the immune function of the body via regulating the maturation process of DC cells (Mazzeo et al., 2020), and the production of proinflammatory cytokines in macrophages infected by *Helicobacter pylori* were also alleviated (Gebremariam et al., 2019). In the present study, it is worthwhile mention that the beneficial bacteria such as *Lactobacillus* and *Lactobacillus gasseri* were the dominant bacteria in the ileum chyme in the 50 SS group. We also found the relative abundance of *lactobacillus gasseri* showed a significant positive correlation with the mRNA levels of *Mucin 2* and *MHC-II* in the ileum, and *ACOT8* in the liver. Additionally, *Lactobacillus* and *Lactobacillus gasseri* showed a negative correlation with the level of serum TG and the mRNA levels of *LxR- $\alpha$*  in the liver. It demonstrated that dietary 50 mg/kg SS might improve the lipid metabolism and intestinal immune function by regulating the structure of the intestinal flora.

In the present study, both levels of SS raised the relative abundance of *Romboutsia* and *Lactobacillus\_pontis*. *Lactobacillus\_pontis* is a bacterium that is highly homologous to *Lactobacillus reuteri* on the phylogenetic tree. It was isolated during the fermentation of green feed and bread. Most of the fermentation products were glucose and oligosaccharide products (Vogel et al., 1994). *Romboutsia* is a type of bacteria that could ferment a variety of carbohydrates and metabolize it to produce SCFAs, oligosaccharides and other prebiotics. Previously, it was reported that *Romboutsia* had a positive correlation with body weight and the levels of blood triglycerides and total cholesterol (Zeng et al., 2019). In addition, the abundance of *Romboutsia* in the intestinal chyme of IBD patients and type I diabetes patients was significantly dropped (Gao et al., 2019; Russell et al.,

2019). Further, *Romboutsia* was closely related to intestinal health (Mangifesta et al., 2018). Since both of these bacteria could ferment carbohydrates, and the relative abundance of both were raised with 2 doses of SS supplementation, both *Romboutsia* and *Lactobacillus\_pontis* could potentially be the SS metabolizing bacteria in the ileum of laying hens. We also found the relative abundance of *Romboutsia* showed a significant positive correlation with the serum TGF- $\beta$  and the mRNA levels of *MHC-II* in ileum. Likewise, beneficial impact of low dose of SS improved the immune function might also be related to the increase in the relative abundance of *Romboutsia*. Taken together, the relative abundance of *Romboutsia* could possibly be an important biomarker to measure the immune function of the intestinal mucosa.

In the present study, with the combined antibiotic treatment, the  $\alpha$ -diversity of the ileal bacteria was reduced, and the intestinal bacterial populations shifted more in favor of harmful bacteria. Specifically, the relative abundance of *Firmicutes*, *Romboutsia*, and *Lactobacillus-gasseri* were reduced. Contrarily, the relative abundance of the *Proteobacteria*, *Helicobacter*, and *Escherichia coli* were elevated. It should be pointed out that the relative abundance of *Romboutsia* was dropped by about 8 times with combined antibiotic treated. We hold the intestinal immunity and barrier function were negatively affected by the changes of these floras. As we found in our study, the ileal villus height, the number of goblet cells, and the ratio of villus height to crypt depth were decreased with combination of tested antibiotics. Beyond that, the level of sIgA in ileum mucosa, the mRNA levels of *MUC-2*, *IL-1 $\beta$* , and *MHC-II* were also downregulated. These evidences elucidated that the immune and barrier function of laying hens was suppressed with combined antibiotics treated. This also illuminated us the importance of intestinal flora in maintaining the intestinal barrier and immune function. It is important to highlight that with combined antibiotic treated; the above-mentioned biological effects of SS basically were eliminated. It further illuminated us that the intestinal bacteria played critical roles in the biological role of SS. At the same time, the bacteria involved in the metabolism of SS might be eliminated. Indeed, potentially beneficial impact of SS in laying hens could be due to the SS metabolites by the intestinal bacteria or the improvement of intestinal flora caused by SS. Further research is needed to clearly understand this mechanism.

Complete ban on the antibiotic usage has greatly changed the production performance and immune function of livestock and poultry. Therefore, it is very important to adjust the immunity of livestock and poultry to an appropriate level. In animal production, due to the anti-nutritional effect of SS, its immune regulation function has often been overlooked. Although a detailed investigation of mechanisms is beyond the scope of this work, we demonstrated that 50 mg/kg SS surprisingly improved the intestinal microflora, the immune function and the lipid metabolism of the laying hens. The results clearly showed the biological role of SS in poultry nutrition.

## CONCLUSION

Dietary 50 mg/kg SS improved the intestinal microflora, the immune function and the lipid metabolism of the laying hens. However, the immune function of the intestinal mucosa was negatively affected with 500 mg/kg SS supplementation. It was noted that the biological role of SS was closely related to the intestinal bacteria. In addition, the long-term use of combined antibiotics caused harmful intestinal bacteria to develop into the dominant bacteria, which induced immunosuppression in laying hens.

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Author Contributions: GY and LP designed the study, LP wrote the manuscript. LP, GM, FJ, YS, and LY collected and analyzed experimental results. LZ and TM participated in the revision of the paper. All authors contributed to the data interpretation and approved the final version of the manuscript.

## DISCLOSURES

The authors declare that there is no conflict of interest.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.psj.2021.101663](https://doi.org/10.1016/j.psj.2021.101663).

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